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Loss of the Acetyl-CoA Carboxylase (*accD*) Gene in Poales

**Mark E. Harris · Gabriele Meyer · Thomas Vandergon ·
Virginia Oberholzer Vandergon**

Abstract The loss of a gene is a rare genome-shaping event
and as such, contributes important information to our un-
derstanding of phylogenetic relationships between genes
and between species. Deletion of a gene can help to define
a lineage. Here, we utilize the deletion of the chloroplast
gene encoding the acetyl-CoA carboxylase subunit D
(*accD*) to help us define lineages based on its presence or
absence in monocot plants specifically in Poales. Southern
blots were constructed and probed for the presence of the
accD gene. The existence of the portion of the *accD* gene
represented by the probe was also verified by PCR and
sequencing. Sequences were utilized for assembly of gene
trees to link the absence or partial loss of the gene with a
particular lineage. Here, we report new information adding
accD gene presence in the Xyridaceae, pseudogene presence
in the Flagellariaceae, and the absence of *accD* in Restio-
naceae and Joinvilleaceae. Based on our findings and the
available data for *accD* sequences in Poales, we propose a
model for *accD* loss beginning with a single event creating a
pseudogene in the common ancestor to the restiid and gra-
minid clades within Poales. This model also suggests that
this pseudogene is carried as the ancestral state throughout
most of the divergence of the Poales, a condition that would
explain the highly varied pattern of *accD* pseudogene pres-
ence or gene absence in members of the restiid and graminid
clades.

Introduction

Ever since the first physical map of chloroplast DNA (maize
1976) and the elucidation of the first full-length chloroplast
nucleotide sequence (*Nicotiana* 1986), the chloroplast ge-
nome has become increasingly valuable for sketching out
the evolution of photosynthetic taxa, especially in land
plants (Clegg 1993; Chu et al. 2003; Sugiura 2003; Givnish
et al. 2010). Studying chloroplast DNA offers many advan-
tages over other genomes for a variety of reasons including
small size and high copy number (Raubeson and Jansen
2005). Much of the phylogenetic significance of chloroplast
sequence data is based on the finding that all chloroplasts
derive from a common ancestor and thus are monophyletic
(Bhattacharya and Medlin 1995). The common ancestor is
the endosymbiont (Margulis 1981) that evolved into the
chloroplast organelle, and its genome has undergone
massive gene number reduction from several thousand
protein-encoding genes (as seen in the putative modern
day ancestral relatives, cyanobacteria) down to between
50 and 250 genes (Melkonian 2001; Martin 2003). It
appears that there existed a common subset of genes in
all chloroplasts with probable positive selection acting
to sustain their usage in modern organisms (Palmer
1985; Barbrook et al. 2006). The conservation of this
subset of genes as well as a slow rate of nucleotide
substitution relative to either nuclear or mitochondrial

M. E. Harris · G. Meyer · V. O. Vandergon (✉)
California State University, Northridge,
18111 Nordhoff St.,
Northridge, CA 91330-8303, USA
e-mail: virginia.vandergon@csun.edu

T. Vandergon
Pepperdine University, 24255 Pacific Coast Highway,
Malibu, CA 90263-4321, USA

genomes are significant because they lend themselves well to evolutionary studies (Wolfe et al. 1987).

Molecular rearrangements in the chloroplast including insertions, deletions, inversions, and transpositions are important because their fixations in the genome occur rarely throughout evolution (Downie and Palmer 1992; Lee et al. 2007). Magee et al. (2010) examined 103 different genomes and found that 27 genes have been lost in at least one lineage. As these events can be marked, the placement of taxa into two groups according to the presence or absence of the rearrangement may define a monophyletic origin. A great opportunity to follow through with defining a monophyletic lineage was undertaken here using loss events of the chloroplast gene acetyl-CoA carboxylase subunit D (accD) as the genome marker. In this paper, accD will always refer to the chloroplast encoded gene.

The accD gene encodes one of four subunits of the acetyl-CoA carboxylase enzyme of the type found in prokaryotes and in most chloroplasts. Acetyl-CoA carboxylase (ACC) is a rate-limiting, catalytic enzyme leading to the formation of malonyl-CoA from acetyl-CoA, the first committed step in fatty acid synthesis (Schulte et al. 1997; Cronan and Waldrop 2002).

The accD gene has been lost either partially or completely from certain monocots (i.e., some members of the Poales and Acoraceae) (Katayama and Ogihara 1996). A multifunctional, nuclear-encoded ACC enzyme has replaced the plastidic enzyme inside the chloroplasts of these organismal groups (Sasaki and Nagano 2004; Cai et al. 2008). Complete sequencing of the nuclear and plastid genomes of some Poaceae species has provided direct evidence of chloroplast accD loss in this family, some of the most derived of all monocots (Chase et al. 2000).

Though the accD gene appears to have been lost in five distinct lineages in dicots, in other dicot lineages, accD has been shown to be essential (Jansen et al. 2007; Magee et al. 2010). For example, in tobacco (a dicot) knockout studies show that accD is essential for proper leaf development and maintenance of plastid structure (Kode et al. 2005). In Arabidopsis, accD was found to be essential for fatty acid biosynthesis and results in embryo lethality if nonfunctional (Bryant et al. 2011). The gene is even preserved in the extremely reduced plastid genomes of the non-photosynthetic, parasitic plant Epifagus virginiana (a dicot) (Kode et al. 2005), and in the underground orchid Rhizanthella gardneri (a monocot) (Delannoy et al. 2011) revealing its probable importance to these organisms.

Martin et al. (1998) and Lee et al. (2007) showed that the accD gene was lost independently in at least five chloroplast genomes, Cyanophora (a glaucophyte sister to red and green algae), Odontella (a diatom in the Stramenopiles sister to the Alveolates), Euglena (protozoa Euglenozoa sister to the Heterolobosea), Oleaceae (a eudicot), and Zea (a

monocot). However, more systematic sampling is necessary to discern whether each loss event was a single occurrence defining a respective lineage or if accD was lost multiple times in each lineage.

This study sought to uncover the point of loss of the accD gene in the Poaceae through more systematic sampling near the putative loss point. Southern hybridization work by Katayama and Ogihara (1996) supported the point of loss of accD in monocots in the progenitor of Poales and Cyperales (based on familial relationships according to Dahlgren et al. 1985). Their data showed no hybridization of an accD probe in 30 species of Poaceae as well as no signal for many species of monocot families very closely related to Poaceae (Katayama and Ogihara 1996). Based on this study alone, the point of accD gene loss appeared to have occurred somewhere before the divergence of Poales from the Commelinales. Another study by Konishi et al. (1996) produced data conflicting with that of Katayama and Ogihara (1996) showing the presence of accD in families Eriocaulaceae, Cyperaceae, and Juncaceae. Our data are consistent with Konishi et al. (1996) but refines the region of loss to a later point of divergence among the Poales.

Most of the monocot families lying in the probable vicinity of the accD loss have no reported gene sequences or are known only from partial sequence data. Therefore, the study presented here was designed to sample more extensively in this phylogenetic neighborhood to clarify the gene loss point in the Poaceae by using Southern hybridization techniques supported by sequence data analysis. As this region of the chloroplast genome (between ribulose–bisphosphate carboxylase (rbcL) and photosystem I (psaI) sandwiching accD) seems to be prone to mutation, it has been dubbed a "hotspot" (Morton and Clegg 1993; Maier et al. 1995; Ogihara et al. 2002). Taking a more detailed look at this region in members related to Poaceae may help define both loss point(s) and potential rearrangement events.

Materials and Methods

Chloroplast Isolation and DNA Extraction

Choice of species for DNA extraction was designed to include monocot families thought to border the putative point of loss of the accD gene and included 24 representative species (Electronic Supplementary Materials (ESM), Table 1). Living specimens were obtained for 23 taxa. Data for taxon, Joinvillea, were performed using total DNA extractions donated by Dr. Cliff Morden of University of Hawaii at Manoa and by Dr. Mel Duvall of Northern Illinois University. All living specimens were maintained at the CSUN Botanic Gardens. DNA was processed from 24 monocot species representing 6 orders and 17 families

(APG 2009) (ESM, Table 1). Eleven species from ten families were sampled across the Poales, and six species were sampled across the Commelinales including five from the family Commelinaceae, two from each major subfamily and one basal representative from the family Haemodoraceae (Burns et al. 2011).

Between 1 and 12 g of leaf tissue were collected as starting material. Fresh material was processed within 30 min of collecting. Isolation of chloroplasts was performed by modification of the protocol by Nobel (1967) with filtered 0.4 M sucrose–Tris–Cl buffer (pH 7.5). Sterile techniques were utilized to avoid bacterial genome contamination as some carry an *accD* gene. After chloroplast isolation, each sample was scanned by light microscopy to verify presence of chloroplasts. This homogenate was filtered and centrifuged to remove whole cells and cellular debris. The remaining suspension of chloroplasts was used in DNA extraction using the Qiagen™ DNeasy Plant Mini Kit. The concentration and purity of extracted DNA were assessed by spectrophotometry.

Polymerase Chain Reaction and Sequencing

Polymerase chain reaction (PCR) amplification primers were designed against conserved portions of the *rbcL* and *accD* gene sequences. Primer sequences are shown in ESM Table 2 and priming sites are diagramed in ESM Fig. 4. The *rbcL* primers were designed for this study by our lab as a control and yielded a product of ~673 bp. Various primers were designed to amplify not only regions within *accD* but also regions up- and downstream ranging from *rbcL* to *ycf4*. PCR amplifications were carried out using a gradient thermocycler (Eppendorf Mastercycler Gradient). Reaction components were generally as follows: both buffer and Taq polymerase from Promega, 2 mM MgCl, 0.05–1.0 μg DNA, 0.42–1.7 μM primers, 0.125 mM dNTPs, and 2 % DMSO. Cycler program was 1 cycle for 2 min at 94°C, 45 cycles (30 s at 94°C, 45 s at 47–55°C, 45 s-4 min at 72°C), and 1 cycle for 7 min at 72°C. PCR products were separated by electrophoresis on 0.8 % TAE agarose.

Products were subcloned using the TOPO TA® Cloning Kit with One Shot® chemically competent Top10 cells (Invitrogen). Plasmids were isolated using Wizard® Plus SV Minipreps DNA Purification System (Promega, no. 1460) and presence of an insert verified by restriction enzyme digestion and gel electrophoresis visualization. Inserts were amplified using M13 primers and sequenced with BigDye™ v.3.1 chemistry on an ABI 377 DNA Sequencer platform located at the CSUN DNA Sequencing Facility. Some PCR products were sequenced directly without the cloning step following gel purification using the MinElute kit (Qiagen, no. 28606).

When internal *accD* primers failed to yield a product, a larger overlapping region was amplified and product size analysis and/or sequencing was utilized to discern one of

three conditions; the presence of the *accD* gene, the partial presence (i.e., similarity to the truncated rice-like *accD* pseudogene), or the total absence of the gene.

Sequence Analysis

Identification of the sequences generated in this study was confirmed by BLAST analysis (Altschul et al. 1990). These new sequences were then aligned with previously sequenced genes downloaded from the NCBI database (ESM, Table 1). Sequences for both genes (*accD* and *rbcL*) were concatenated and aligned by ClustalW (Thompson et al. 1994) using the free software BioEdit version 7.0.0 (Hall 1999) and edited by eye. The data matrix consisted of 1,164 nucleotides for 43 species (39 sequences for *accD* (527 bp+14 binary) and 43 sequences for *rbcL* (637 bp)). Of the four missing *accD* sequences, one was coded as a deletion verified by sequence data and the others were conservatively coded as missing data because no PCR products were obtained in repeated attempts preventing us from independently verifying the absence of the gene in these species. Gaps were coded as binary data at the end of each sequence block for Bayesian analysis. Two approaches were taken for sequence analysis: maximum likelihood using PAUP*4.0 (Swofford 2002) and Bayesian inference using MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003; Huelsenbeck and Ronquist 2005). The bootstrapping technique was applied using PAUP with "fast" stepwise addition for 500 replicates to test node support. For Bayesian analysis, 3 million generations were run and best gene tree was constructed minus 25 % burning (Fig. 3).

Southern Blotting

Approximately 10-μg template DNA from each of 21 species was digested with either EcoRI or HindIII. Digestions were cleaned and concentrated by ethanol precipitation and then run on a 0.7 % TAE agarose gel. Blotting was done by upward capillary alkaline (NaOH) transfer onto a positively charged nylon membrane (Boehringer Mannheim, no. 1417240) and set by baking 1 h at 99°C.

To produce DNA clean enough to be labeled for use as probes for Southern blotting, PCR products were obtained from amplification of *Callisia fragrans* (Commelinaceae) DNA using the accDf2′/R primers and amplification of *Dichorisandra thyrsiflora* (Commelinaceae) DNA using rbcLR1/F1 primers. These products were then subcloned, extracted, and purified by gel extraction with a MinElute gel extraction kit (Qiagen, no. 28606). The purified products were quantified by spectrophotometry. This probe template was labeled with biotin in a one-step process using the *Label*IT kit (MIRUS, MIR3400). Hybridization and detection of genomic DNA were performed with the North2-South® Chemiluminescent Hybridization and Detection

Kit (Pierce, no. 17097). Hybridization time varied between 8 and 12 h. Stringency washes consisted of 0.1 % SDS/ 0.1× SSC and 10–30 % formamide at 65°C. Blot exposure to X-ray film (Kodak X-Omat FS-1) varied from 10 min to 1 h. Blots were first probed with the *accD* probe then stripped and re-probed with the *rbcL* probe. Stripping was accomplished by incubation at 37°C with gentle shaking in 0.2 M NaOH/0.1 % SDS solution for 30 min. Blots were stored at 4°C in 2× SSC between hybridizations.

Results

Evidence for the presence/absence of the *accD* gene was shown by PCR amplification, sequencing and/or by Southern blot hybridization (Fig. 1). *AccD* in these species lies in

a chloroplast segment between the *rbcL* (ribulose carboxylase large subunit) and the *ycf4* gene (an essential gene for photosystem I assembly) (Ozawa et al. 2009). The order of genes in this region of the chloroplast is *rbcL*, *rpl23*, *ORF133*, *accD*, *psaI*, and *ycf4* (Fig. 2). Variations in this pattern were used in developing a model for *accD* loss (see "Discussion").

Southern Blotting

Southern blot hybridization provided positive/negative scoring of taxa for both the *accD* and *rbcL* genes. Hybridization was positive for *accD* in all species tested except *Joinvillea* and *Bambusa*, consistent with our expectation that the gene is absent or truncated in these two groups (see "Polymerase Chain Reaction" section). Furthermore, the *rbcL*-positive

order	family	genus species	PCR product		sequence obtained		Southern blot		accD gene
			accD	rbcL	accD	rbcL	accD	rbcL	
Brassicales	Brassicaceae	Brassica napus	+		+				Yes
Asparagales	Asparagaceae	Chlorophytum comosum	+		+		+	+	Yes
		Lomandra longifolia	+		+		+	+	Yes
	Hyacinthaceae	Veltheimia bracteata	+		+				Yes
Arecales	Arecaceae	Brahea armata	+	+	+	+	+	+	Yes
Zingiberales	Strelitziaceae	Strelitzia nicolai	+	+	–		+	+	Yes
Commelinales	Haemodoraceae	Anigozanthos hybrid	+	+	+		x	x	Yes
	Commelinaceae	Callisia fragrans	+	+	+		+	+	Yes
		Commelina communis	+		+		x	+	Yes
		Palisota ambigua	+		+				Yes
		Tradescantia virginiana	+	+	+	+			Yes
		Tradescantia zebrina	–	+			+	x	Yes
Poales	Bromeliaceae	Tillandsia secunda	+	+	+		+	+	Yes
	Typhaceae	Typha latifolia	+	+	+		–	–	Yes
	Cyperaceae	Carex comans	+	+	+		+	+	Yes
	Juncaceae	Juncus effusus	+	+	+		+	+	Yes
	Eriocaulaceae	Eriocaulon sp.	+	+	+				Yes
	Xyridaceae	Xyris caroliniana	+	+	+		+	+	Yes
	Flagellariaceae	Flagellaria indica	+	+	+	+	+	+	Yes
	Restionaceae	Baloskion tetraphyllum	–	+					No
		Chondropetalum tectorum	–	+			x	x	No
	Joinvilleaceae	Joinvillea plicata	–	+	deleted		–	+	No
	Poaceae	Bambusa valida	–	+	pseudo		–	+	pseudo
		Oryza sativa	–	+	pseudo		+	+	pseudo
		Sorghum bicolor	–	+	deleted				No

Fig. 1 Results of Southern blot, PCR and sequence analyses conducted in this study and used to determine *accD* gene status. *Boxes containing minus sign (−)* denote no PCR band/no sequence of a PCR product or no Southern blot signal. *Boxes containing plus sign (+)* denote presence of a PCR product of expected size, product was sequenced or a positive signal in Southern blotting. *Boxes containing letter x* denote unreadable Southern blot data. *Shaded boxes* denote tests not performed
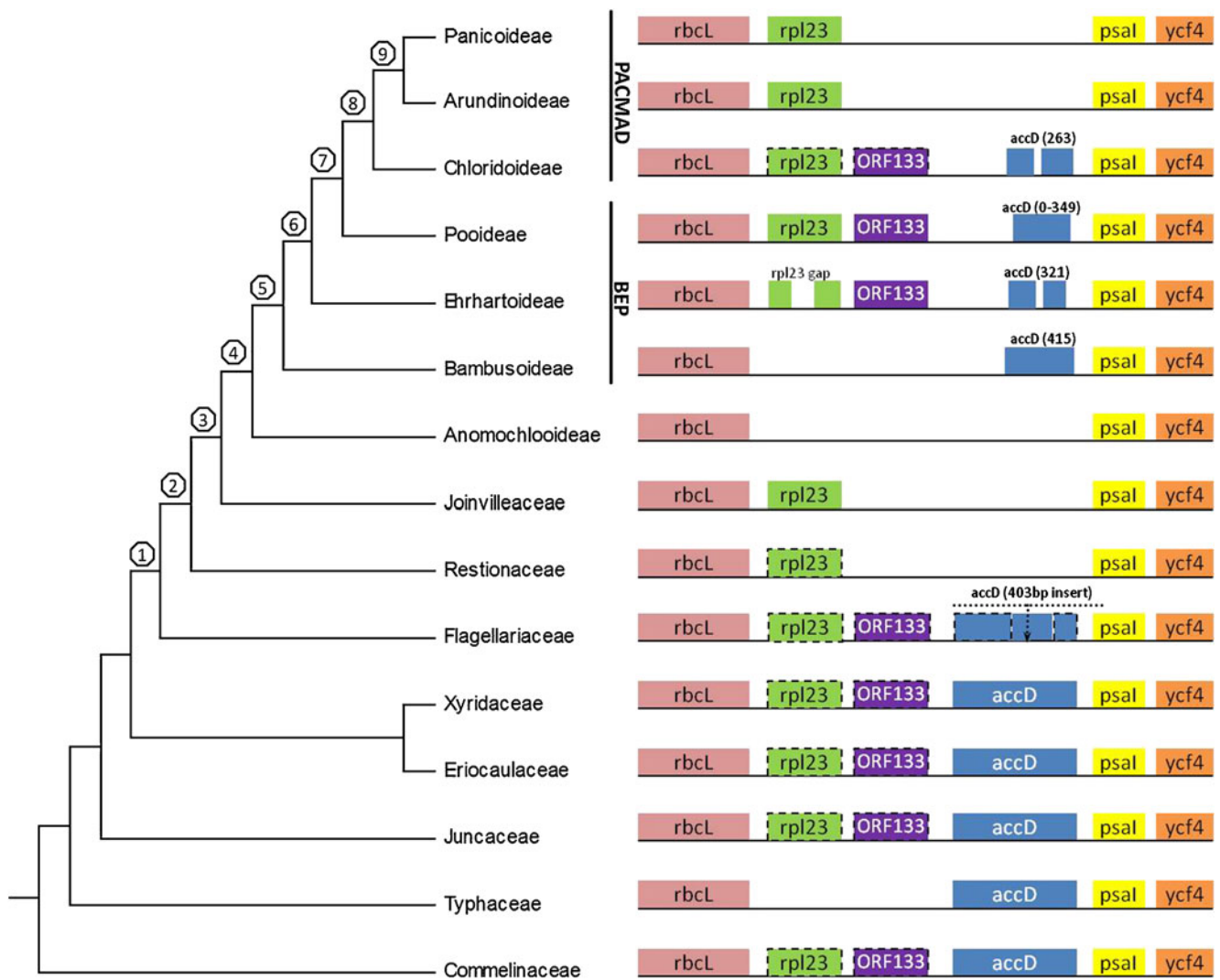
Fig. 2 Overview of the *accD* gene presence in monocot families from Commelinaceae through Poaceae. Gene layout is not to scale. *Numbers in parentheses* for *accD* pseudogenes indicate known number of basepairs either published or from this study. *Dashed lines* indicate uncertain gene presence. The figure assumes the *psaI* and *ycf4* genes are present in all families shown although we did not confirm this in all groups. The presence of a full copy of *accD* is assumed for Commelinaceae, Juncaceae, Eriocaulaceae, and Xyridaceae based on high identity mid-gene coverage data obtained in the current study. *Numbers at nodes* represent the ancestral states and are explained in text. The *model tree* presented to the *left* represents an amalgam of family relationship understanding from published analyses (Givnish et al. 2010; Morris and Duvall 2010) and the gene loss events portrayed here

control probe hybridized both of these species. *Oryza* and *Bambusa* carry only pseudogenes of *accD* (Hiratsuka et al. 1989; Wu et al. 2009) that differed in probe specificity. The *accD* probe was able to bind sufficiently to *Oryza* but could not detect the *Bambusa* pseudogene. The *rbcL* probe produced a much stronger signal in *Oryza* compared to *Bambusa* indicating that lack of signal may have been due to low concentration of *Bambusa* DNA.

Species that gave positive PCR and sequencing results for both genes also showed clear hybridization with the probes with few exceptions (Fig. 1). In these cases, gene sequencing was deemed sufficient to determine presence in these genomes. The Southern data for *Chondropetalum*, *Typha*, and *Anigozanthos* were not reliable and so are not considered here. *Typha* and *Anigozanthos* produced PCR products and *accD* sequences as expected. *Chondropetalum*, however, produced neither PCR product for *accD* in repeated amplification attempts (as evaluated by gel electrophoresis) nor sequence data of the gene. PCR with *rbcL* primers was the only test to give positive results for *Chondropetalum* indicating the presence of *rbcL* and that the DNA was reliable.

Polymerase Chain Reaction

Amplification of *accD* was attempted for all species and the product was successfully subcloned and sequenced for most. Taxa for which exhaustive PCR never resulted in the *accD*

product were tested using *rbcL* primers to ensure that the DNA template was adequate for amplification. The *rbcL* primers produced a band of the expected size for all species attempted (Fig. 1). Most *rbcL* PCR products were not sequenced because the *rbcL* sequences for those species were available through Genbank. Taxa *Baloskion*, *Chondropetalum* (both of family Restionaceae), and *Joinvillea* (family Joinvilleaceae), which are closely related to Poaceae (APG 2009), never amplified *accD* in repeated attempts. All species producing an *accD* PCR product produced readable *accD* sequence except for *Strelitzia nicolai* (family Strelitziaceae, order Zingiberales), and there is no reason to suspect that the *accD* gene does not reside in *Strelitzia*.

Primers designed to amplify the sequence between *rbcL* and any of the following genes *accD*, *psaI*, or *ycf4* worked well in both control species: *C. fragrans* (family Commelinaceae, contains whole *accD* gene) and *Oryza sativa* (family Poaceae, contains truncated, *accD* pseudogene). By comparison of test products to control products, we were able to distinguish between absent genes, putative pseudogenes, and probable full-length genes. Figure 2 shows a summary map of the *rbcL* to *ycf4* gene region in the monocot families from the Commelinaceae through the Poaceae outlining gene status.

All monocot families outside of the Flagellariaceae possessed full-length *accD* copies. Although our accDf2′/accDR primers amplified only a part of the entire gene, both *Eriocaulon* and *Xyris* likely carry the complete *accD* gene based on the high level of conservation of the sequence data obtained for each species. Interestingly, while the relative positioning of genes from *rbcL* to *ycf4* are the same as in later lineages *rpl23* and *ORF133* sequences present in many Poaceae are absent here. The gene region between *rbcL* and *accD* is known only from *Typha* (Typhaceae) (Jansen et al. 2007; Guisinger et al. 2010) but this region is assumed to be the same (except in Joinvilleaceae—see next) in this part of the lineage for all the Poales basal to the Poaceae. The *rpl23* translocation has been studied by Katayama and Ogihara (1996) who report *rpl23* only in the Poaceae; here, we add the novel presence of *rpl23* adjacent to *rbcL* in the Joinvilleaceae.

Flagellaria (Flagellariaceae) carries a fragmented version of *accD* that is likely a pseudogene. In the sequence, between the primers accDf2′—accDR, *Flagellaria* contains one insertion of 403 nucleotides (nt) not found in any other species tested so far. In addition, there is an early stop codon in the coding region just before the insertion. Sequences homologous to *rpl23* or *ORF133* were not found immediately downstream of *rbcL* in *Flagellaria*. It should be noted that there was some sequence upstream of *accD* that did not get fully sequenced but based on size of the PCR product, it does not seem likely that these sequences are present.

Repeated attempts to amplify *accD* from representatives of the Restionaceae (*Baloskion* and *Chondropetalum*) produced no PCR products even though the *rbcL* gene was present and amplified. Southern blot data for these species yielded unusable results. We concluded from these efforts the likely absence of *accD* in the Restionaceae. While *Baloskion* and *Chondropetalum* produced *rbcL* PCR products and/or sequences, extensive PCR/sequencing covering the region between *rbcL* and *ycf4* did not produce any sequences homologous to *rpl23*, *ORF133*, or *psaI*, a scenario similar to the missing *accD*. However, like *Flagellaria*, there remain some unsequenced sections.

In *Joinvillea* (Joinvilleaceae) complete sequencing across the region between *rbcL* and *ycf4* showed a full-length *rpl23* gene but no evidence for either *ORF133* or *accD* not even a pseudogene. We conclude that *accD* is completely lost from this group.

The gene region spanning the *accD* in members of the Poaceae is more complicated. The purported basal subfamily Anomochlooideae (Givnish et al. 2010; Morris and Duvall 2010) in thorough sequence analysis showed the complete absence of *rpl23*, *ORF133*, and *accD* despite the fact that more derived members of the Poaceae possess either complete copies or fragments of these genes.

Sequencing *Bambusa* (Bambusoideae) DNA across the entire region between *rbcL* and *ycf4* revealed a pseudogene of *accD* 415 bp in length. In addition, no sequence evidence for *rpl23* or *ORF133* was detected. *Bambusa* is part of the well-supported BEP clade (subfamilies Bambusoideae+ Ehrhartoideae+Pooideae) (GPWG 2001) which is sister to the PACMAD clade (Panicoideae+Arundinoideae+Chloridoideae+Micrairoideae+Aristidoideae+Danthonioideae) within the Poaceae (originally, PACCMAD, Sanchez-Ken et al. 2007; Duvall et al. 2007; Sanchez-Ken and Clark 2010).

Comparison of published or submitted sequences in the *rbcL* and *ycf4* region from members of the two other BEP subfamilies also showed the presence of truncated *accD* pseudogenes and variable presence of *rpl23* and *ORF133* genes. The *accD* pseudogene from *Oryza* (Ehrhartoideae) is similar to that in *Bambusa* but has experienced the loss of an additional 78-nt stretch in the middle of the fragment and a 20-nt stretch at the 3′ end of the fragment (Hiratsuka et al. 1989). Several representative members of the Pooideae (e.g., wheat, rye and barley) show variation in partial *accD* gene presence, ranging from 0-nt (absent) to a 349-nt pseudogene fragment (Ogihara et al. 2000, 2002; Aagesen et al. 2005; GI accession nos. 164453499 and 118201020). Within the BEP clade, the region upstream of the *accD* pseudogene region shows a wide range of conditions. Members of the Pooideae appear to have full-length *rpl23* and *ORF133* sequences whereas *Oryza* (Ehrhartoideae) bears a *rpl23* pseudogene with a full-length *ORF133*.

Among the known sequences in members of the PAC-MAD clade, *Eragrostis* (Chloridoideae) has a known *accD* pseudogene (Kress and Erickson 2007) while members of the Arundinoideae and Panicoideae show no sign of *accD* (Chu et al. 2011). The *rpl23* gene is present in most PAC-MAD groups but *ORF133* seems to be absent in many.

Phylogenetic Trees

Phylogenetic analyses were conducted on the dataset with *accD* + *rbcL* sequences combined using two methods, maximum likelihood and Bayesian analysis yielding the phylogenetic tree shown in Fig. 3. The gene tree showed distinction of the outgroups (hornwort *Anthoceros* and whisk-fern *Psilotum*)

and clustering of species more or less agreed with traditional taxonomy of angiosperms, including dicot and monocot groupings. Clade support values among the tree nodes are generally low (below 95 %) with some exceptions as noted. Low support was expected here given the small data set and the specific goal of examining *accD* relationships in the order Poales. The gymnosperm clade was well supported in both analyses and the magnoliid clade was conserved but not given significant support in either analysis. The separation between monocot and dicot clades was also preserved (Fig. 3). The Bayesian prior probabilities (only) support the delineation between the Liliopsida (monocots) and eudicots with the magnoliids basal to both. The order Poales separated as expected from the order Commelinales with *Brahea* (order Arecales) as

Fig. 3 Best tree constructed from dataset *accD* + *rbcL* by two phylogenetic estimation methods. *Numbers near nodes* indicate support (percent of 500 bootstrap replicates by Maximum Likelihood (*top number*) and posterior probability values based on 3 million generations sampled every 100th generation by Bayesian analysis (*bottom number*)). The *solid line box* encloses the dicot clade. The *dashed box* encloses species associated with *accD* gene loss (see "Discussion")
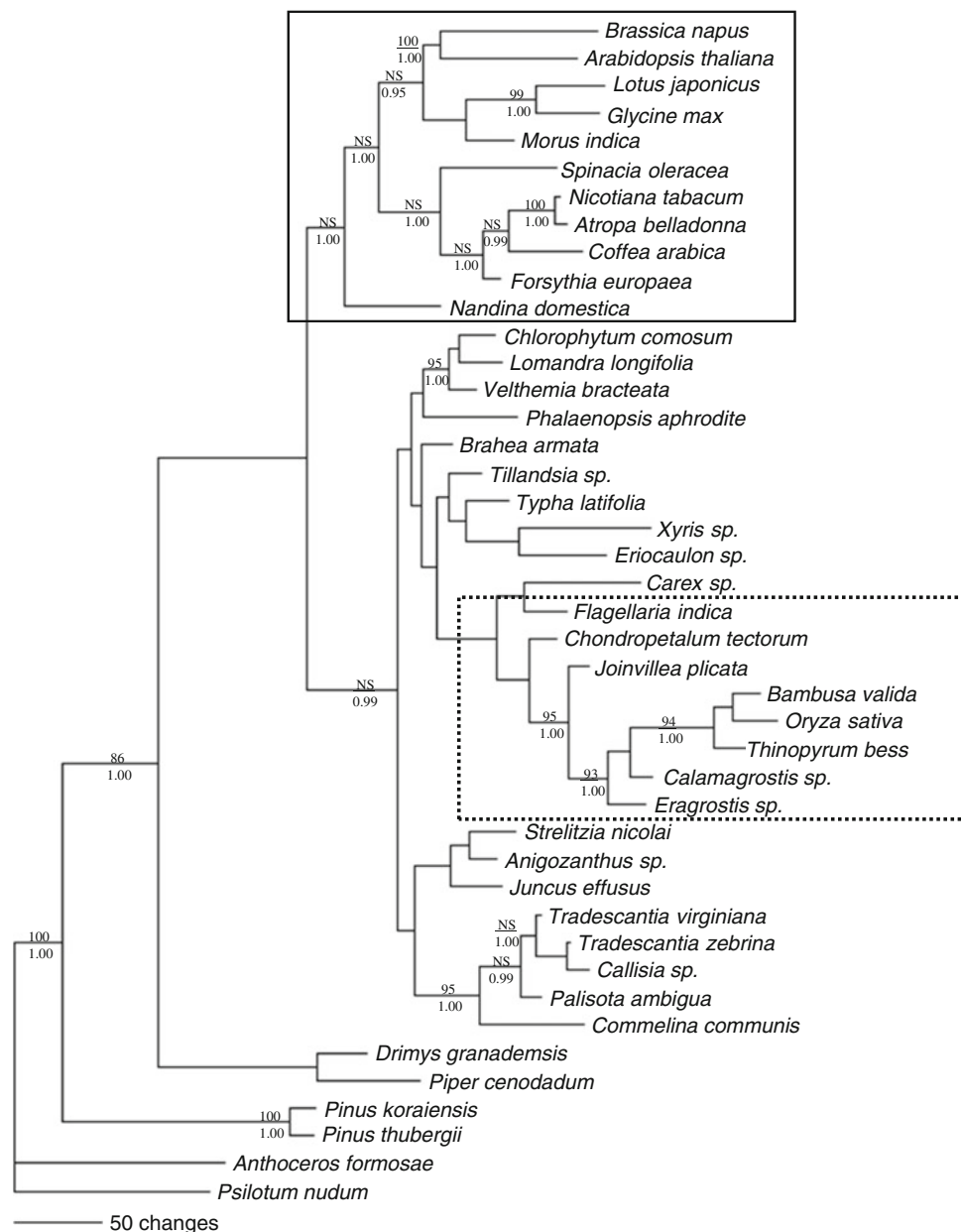
basal to the Poales. The only exception to this was *Juncus* (a Poales) positioned inside the Commelinales group. Within the Poales, there are a few supported arrangements notably *Joinvillea* as basal to the PACMAD representative *Eragrostis* and the BEP clade (*Calamagrostis*, *Thinopyrum*, *Oryza*, and *Bambusa*). Our results place *Chondropetalum* (a Restionaceae) clustering outside of *Joinvillea* but inside *Flagellaria*, an unusual placement relative to published work (see "Discussion") (Bremer 2002; Givnish et al. 2010).

Discussion

Gene Loss

While the ideal method to identify a loss point for *accD* would be to analyze the whole-genome chloroplast (cp) sequences of all the Poales, lack of whole-genome cp sequence data covering all Poales restricts this study to alternative methods including Southern blot analysis and PCR sequencing to determine loss events among a wide array of monocots. Our Southern blot results were intended as the first line of evidence for gene presence or absence. Negative Southern results using the *accD* probe were further investigated by PCR and verified by sequencing where possible of the *accD* gene and/or the region between the genes *rbcL* and *psaI*, flanking *accD* in 13 representatives of the Poales and six related plant orders (Fig. 1).

To our knowledge thus far, there are three reported occurrences of *accD* pseudogenes, all occurring in the Ehrhartoideae (*Oryza, Potamophila and Microleane*) (Nock et al. 2011). Here, we report an *accD* pseudogene in *Bambusa*, similar to that in *Oryza* but longer (Fig. 2). Also, in analyzing the deposited whole-chloroplast genome sequences from the BEP clade of Poaceae, the presence of the *Oryza*-type *accD* pseudogene was revealed in the Pooideae ranging from 0 bp in at least one species of Triticeae (Ogihara et al. 2002) up to 349 bp in *Secale* of the Triticeae (Aagesen et al. 2005) (Fig. 2). We also report the absence of *accD* from Restionaceae and Joinvilleaceae and the presence of an *accD* pseudogene in the Flagellariaceae. The only PACMAD representative known to have a portion of *accD* is *Eragrostis* (in sub-family Chloridoideae) which carries a 263-bp pseudogene (Kress and Erickson 2007).

Our data clarifies the two published reports for loss points of *accD* and points to a node of initial loss. Katayama and Ogihara (1996) placed the *accD* loss point prior to the divergence of the Poales. Konishi et al. (1996) found *accD* in Eriocaulaceae, Cyperaceae and Juncaceae. Here, we add *accD* gene presence to the Xyridaceae and partial gene presence in Flagellariaceae. Our new data combined with additional sequences from Genbank deposits have allowed us to propose a model for *accD* loss at a point in the

evolution of Poales past the Eriocaulaceae/Xyridaceae split (see Bremer 2002 and Givnish et al. 2010).

Model of *accD* Loss

Our data show several important mutational event positions that inform us about *accD* loss as indicated by node numbers on the model presented on the left in Fig. 2. The common ancestor to the Flagellariaceae and later families in the monocots reveals that a first mutational event corresponding to a loss of function in *accD* occurred at node one. All known members past this point have no possible plastid *accD* function even if sequence is present. The next two nodes (2 and 3) lead to (in our representation) the families Restionaceae and Joinvilleaceae. These two groups (along with the Flagellariaceae) are clearly basal to the Poaceae (Bremer 2002; Givnish et al. 2010) but this position also represents a place of potential ambiguity. Our loss model moves the Restionaceae inside of the Flagellariaceae only for the purpose of defining the node of loss (discussed later). None of the sequence matching that of the *accD* probe was found in *Joinvillea* (Joinvilleaceae), *Chondropetalum*, and *Baloskion* (Restionaceae) by us, meaning that the ancestral pseudogene was lost in these lineages. These data correspond with phylogenetic data that clusters the Joinvilleaceae with the Poaceae, the grass family, and preserves the Graminid clade which also lacks the *accD* gene (APG 2009; Bremer 2002). Our phylogenetic analyses agree with this too (see below).

If we assume a functional loss of *accD* by insertion or truncation or both creating a pseudogene in *accD* in the common ancestor at node one, the variable presence of a pseudogene in the BEP clade and some members of the PACMAD clades (Chloridoideaea) is easily understood, and strongly supports the argument that the common ancestor at nodes 2–8 probably possessed a pseudogene as well. The extent by nucleotide count to which the *accD* gene is present in each of the three subfamilies of BEP infers gradual loss with the most advanced in Pooideae and the least lost in Bambusoideae with the retention of a very truncated version in one subfamily of PACMAD. This correlates with other findings that among the BEP, Pooideae has the closest relationship to the other major subclade of Poales, the PACMAD clade (GPWG 2000) most of which do not carry any recognizable remnants of *accD*.

This model would lead us to propose that the remnant pseudogene originating at node one and present through nodes 2–8 was subsequently lost several independent times along the lineages for the Restionaceae, Joinvilleaceae, and Anomochlooideae and the ancestor at node eight in the PACMAD clade. The alternative hypothesis to our model would necessarily invoke a complete loss in three lineages past node one (perhaps at node two) with a reinsertion of a

pseudogene in the ancestor to the BEP clade with another event loss at node eight. While this may appear to be a viable option, the mechanism and source of a pseudogene reinsertion to us is problematic. Within the BEP clade and an early member of the PACMAD clade, we can see evidence of both gradual and complete loss of *accD* following three lineages of absence; very possible if all had an ancestral pseudogene. Our proposal here of an initial functional loss in the ancestor at node one followed by a varied pattern of complete and partial additional losses of sequences at later node points seems logical. We know that this region of the plastid genome is a hotspot for mutational activity (Ogihara et al. 1991; Morton and Clegg 1993; Maier et al. 1995) causing us to believe that multiple independent and sometimes confusing loss/rearrangement patterns might exist.

A confusion of independent losses occurs in the BEP/ PACMAD clades of the Poaceae because the Ehrhartoideae include both the *rpl23* and *accD* pseudogene sequences each interrupted by missing segments while Bambusoideae (sister group to the Ehrhartoideae) does not contain any of the *rpl23* sequence but does contain a non-segmented and lengthier sequence of *accD* (Fig. 2). *Triticum aestivum* is the only member of the BEP (specifically Pooideae) found so far not to carry any sequence of the *accD* gene while *Triticum monococcum* is found to contain a truncated *accD* gene. The available data to date show all Pooideae species possessing *rpl23*. In addition, *rpl23* exists in members of the PACMAD which (with one known exception) are completely missing *accD*. So, deletions in the BEP subfamilies (Fig. 2) may or may not have been independent events. Clearer understanding will depend on whether or not the Pooideae are sister to Bambusoideae and Ehrhartoideae or to the PACMAD, a yet uncertain relationship (Kellogg 2000; Saarela and Graham 2010).

The serial loss of *accD* in Poales described here shows similar characteristics to the serial loss of *accD* in the dicot tribe Jasmineae (Lee et al. 2007). In the Jasmineae, the 5′ and 3′ ends were lost before the middle of the gene. Our data suggest a similar scenario in bamboo, rice, the Pooideae, and the PACMAD, indicating that loss began at the extremities of the gene sequence (Fig. 2).

Phylogenetic Implications of the *accD* Loss Model

Our *accD* data were combined with *rbcL* data generated here and from database information to use in phylogenetic analysis thereby testing our loss model. Clearly, resolution of the true point of loss of the *accD* gene will require more substantial sampling than our dataset provides, however our tree results appear to support our model (Fig. 3).

Our phylogenetic analysis using the *rbcL* and *accD* data mirrors the model arrangement of basal monocot orders. Orders Asparagales, Zingiberales and Commelinales cluster in two loose groups but the Poales are clearly defined and are correctly derived from the Arecales (Givnish et al. 2010) where chloroplast gene order similar to the basal Commelinids is preserved (Khan et al. 2011). Our branching, not unexpectedly, follows the pattern of proposed loss of *accD*. This pattern reflects the possession of either a full-length gene, a pseudogene or absence of the gene. Only two oddities arise. The first is the positioning of *Carex* (a Cyperaceae) and *Juncus* (a Juncaceae) in sister families (Bremer 2002; Givnish et al. 2010) that both possess probable full-length *accD* genes. These two split to odd positions near Flagellariaceae (*Carex*) and within the Commelinales (*Juncus*). We can only assume that these positions are artifacts of sequence identity in the *rbcL* or *accD* genes. Because both of these species lie outside of the node point of initial *accD* loss, while positioned improperly, do not impact our refinement of the *accD* loss point in the Poales.

The second oddity in our tree is the relative position of the Flagellariaceae and Restionaceae (Fig. 3). Our data, tree, and model place Flagellariaceae outside of the Restionaceae whereas phylogenetic analyses using multiple plastidic gene data sets (Saarela and Graham 2010; Givnish et al. 2010) reverse these positions. We are not refuting that arrangement here. The data that switch these positions in our small data set almost certainly lies within the *accD* portion of the sequences which naturally would draw the Flagellariaceae pseudogene data closer to the full-length functional sequences in the other Poales over the lack of any *accD* sequence for the Restionaceae. Our tree positioning actually would be predicted by a model where the Flagellariaceae bears the closest relationship to the basal ancestor to both the restiid and graminids. We propose *that* ancestor as bearing the new *accD* pseudogene.

Loss of a gene is not always a reliable indication of phylogenetic relationships as seen in the apparent multiple independent losses of chloroplast genes *rpl22, infA, rps16* (Jansen et al. 2006) and even the gene of our study, *accD*. The *accD* gene has been deleted independently in many chloroplast-carrying lineages other than the Poales (Martin et al. 2002; Chumley et al. 2006; Maier et al. 1995; Goremykin et al. 2005; Lee et al. 2007; Cosner et al. 2004). Also, independent partial deletions have occurred in non-angiosperms, such as in liverwort (a nonvascular plant), and black pine (gymnosperm). One explanation for the common occurrence of deletions is that *accD* lies in a region of high mutation rates, termed a "hot spot" (Ogihara et al. 1991; Morton and Clegg 1993; Maier et al. 1995). Its position in the chloroplast genome also appears to be associated with a rearrangement endpoint as found in the dicots *Pelargonium*, *Trachelium*, and the Lobeliaceae (Chumley et al. 2006). These findings support the probability of independent partial deletions among the Poales as described above.

As pointed out by the Angiosperm Phylogeny Group III (APG 2009), the order Poales, although accepted as being

monophyletic, still lacks clarity of family interrelationships. For instance, Joinvilleaceae is tentatively found by APG III to be sister to Poaceae while Bremer (2002) found strong support for Ecdeiocoleaceae as sister. Our data suggest that Joinvilleaceae is closest to Poaceae among our set of tested families, which unfortunately did not include Ecdeiocoleaceae. Ecdeiocoleaceae, therefore, is a family that should be tested for the presence of the *accD* and *rpl23* genes and may help in the resolution of the true sister to Poaceae.

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